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Sequential Differentiation of Sexual Behavior in Populations of *Drosophila silvestris*[1]

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ABSTRACT: Laboratory strains from six widely distributed populations of *Drosophila silvestris* from the island of Hawaii were established. Mate preference experiments were conducted to determine whether behavioral differences were present among the six populations. In nearly all pairwise combinations studied, at least partial (asymmetrical) isolation was observed between reciprocal crosses; i.e., females from one population were less discriminant than females from the other. Kaneshiro (1976, 1980) has hypothesized that the genetic basis of certain elements of the courtship behavior pattern in these species change (are lost) such that females of ancestral populations discriminate strongly against males of derived populations, while females of derived populations readily accept males of ancestral populations. Following such an hypothesis, the data obtained from this study provide a basis for inferring the direction of evolution among the six populations of *silvestris*. It would appear that the population on Hualalai is the oldest population, and from there, two separate lineages gave rise to the remaining five populations. One lineage provided progenitors for the south and west populations, i.e., at Pauahi and Kahuku. The second lineage involved an introduction from Hualalai to the Kohala Mountains and subsequent colonizations of the Piihonua and Olaa populations. When interpreted in this way, the behavioral studies appear to be a sensitive indicator of initial stages of the speciation process.

THE DYNAMICS OF SPECIATION may be extrapolated from studies of two or more closely related but full biological species as well as from studies of any single interbreeding species. However, clear inferences about the speciation process can best be realized through examination of incipient species, i.e., species in *statu nascendi*. Although evolution is a continuous process, such examples of incipient speciation are rarely discovered, since it is believed that the dynamic stages of genetic divergence between two populations are of relatively short duration (Richmond and Dobzhansky 1976). One of the best such examples in the genus *Drosophila* is the superspecies *paulistorum*, consisting of six semispecies. Numerous studies of reproductive isolation among these six populations have been reviewed by Dobzhansky and Powell (1975).

More recently, Carson and Bryant (1979) report on a newly discovered morphological character in some populations of *Drosophila silvestris* on the island of Hawaii and suggest that such "a new embellishment of a secondary sexual character" is evidence for incipient speciation. The character these investigators discuss is the presence of an irregular extra row of cilia on the dorsal surface of the front tibia in *silvestris* males. The addition of the extra, or third, row of cilia is believed to be a recently evolved character, because the closely related species *D. differens* and *D. planitibia*, which are found on geologically older islands (Molokai and Maui, respec-

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tively) and are believed to be ancestral to *silvestris* (Kaneshiro 1976), lack this character. It has thus been proposed that the populations of *silvestris* on the north and east sides of the island of Hawaii acquired the new morphological (secondary sexual) character as a result of behavioral differences acquired after allopatric speciation from populations on the south and west sides of the island, which also lack this extra row of cilia.

In a study of sexual isolation among four species of Hawaiian *Drosophila*, Kaneshiro (1976) observed that females of ancestral populations strongly discriminated against males of derived populations, while females from derived populations accepted males of ancestral populations as well as males from their own population. Kaneshiro (1976, 1980) hypothesized that this phenomenon of one-sided mating preference could be explained if, during the flush/crash cycles of the speciation theory proposed by Carson (1968, 1971), the observed behavioral change is the result of a less complex mate recognition system in the newly founded population; i.e., changes occur in the courtship pattern of derived populations which lower the receptivity threshold of the females.

Ohta (1978) analyzed sexual isolation among six populations of two other closely related Hawaiian *Drosophila* species. By applying the hypothesis proposed by Kaneshiro (1976, 1980), he was able to formulate a unidirectional phylogeny for the six populations. The phylogenetic relationship based on the interpretation of the behavioral data was consistent with the geological ages of the islands on which the six populations are found; i.e., the evolutionary sequence of the origin of the six populations studied showed that the ancestral population was found on the oldest island, while the most derived population was found on the youngest island of the Hawaiian Archipelago.

Bicudo (1978) used the same hypothesis to interpret similar kinds of sexual isolation data obtained from crosses among geographical populations of *Drosophila prosaltans* in Brazil and Central America. She also was able to propose a unidirectional phylogeny that appears to be consistent with phylogenies proposed on the basis of cytological and hybridization data. She showed that populations of *prosaltans* in Brazil were ancestral and those from Central America were derived.

Kaneshiro (1980) reviews some of the literature on sexual behavior in *Drosophila* in which the investigators observed similar kinds of asymmetrical behavioral differentiation between populations. The most convincing studies that provide further support of the Kaneshiro hypothesis were reported by Arita and Kaneshiro (1979) and Powell (1978). In the former, two strains of a Hawaiian *Drosophila* species were studied using mate preference experiments. Again, strong asymmetry was observed between the two reciprocal combinations. The authors speculated that the females of the older strain, which had been subjected to several unintentional genetic drift situations (i.e., population crashes) while in the laboratory, became less discriminant because of changes (loss) in the genetic basis of certain behavioral elements in the mate recognition system. In Powell's study, laboratory populations of *D. pseudoobscura* were intentionally subjected to four flush/crash episodes in an experiment designed to test the founder/flush speciation theory. In three of the eight lines of *pseudoobscura* studied by Powell, striking behavioral asymmetry was observed when compared with the stock that was considered to be ancestral. In all three cases, it appeared that females from the derived strains had become less discriminant than females from the ancestral stock. Again, the data seem to suggest that the drift conditions resulting from the crash episodes affected the mate recognition system of the derived strains so that such females are less discriminating than females from ancestral strains.

It is appropriate at this point to present the views of Watanabe and Kawanishi (1979), who have made similar observations of asymmetrical behavioral differentiation between populations of *Drosophila*. Watanabe and Kawanishi have chosen to interpret their data in such a way that the direction of evolution of the species they studied is exactly the opposite to the direction inferred by application of Kaneshiro's hypothesis. These investigators claim that "It may be

TABLE 1

LOCALITIES ON THE ISLAND OF HAWAII OF THE SIX *Drosophila silvestris* POPULATIONS USED IN THIS STUDY

SITE NO.	LOCALITY	STOCK NO.
1	Kaupulehu, Mt. Hualalai, 4600 ft elevation	U51Y32 (standard)
2	Waiopae, Kahuku Ranch, Kahuku, 4100 ft elevation	U26B9 (standard)
3	Pauahi, Greenwell Ranch, Kona, 4400 ft elevation	U61 (16 strains)
4	Puu Laalaau, Kohala Mts., 4000 ft elevation	U34B4 (standard)
5	Olaa Forest Reserve, Kilauea, 3800 ft elevation	T94B7 (standard)
6	Piihonua, Saddle Road, Mauna Kea, 4140 ft elevation	U57 (12 strains)

pointed out that the evolutionary sequence given by our hypothesis agrees with the established phylogenies in *Drosophila* groups" (1979 : 907). The "established phylogenies," which they claim corroborate their behavioral phylogeny, are based on cytological, morphological, and electrophoretic analyses. In nearly all cases, investigators who use these methods to propose phylogenies recognize the shortcomings of such phylogenies in that *polarity* is a serious question frequently left unanswered. The directionality of phylogenies based on cytology, morphology, or electrophoretic analyses is usually based on an arbitrary standard that has been selected only for convenience. The evolutionary sequence of a group of species based on such data could be proposed in either direction with equal probability. Furthermore, the reports by Arita and Kaneshiro (1979), Ahearn (1980), and, especially, Powell (1978) provide strong experimental evidence against Watanabe and Kawanishi's hypothesis while supporting that proposed by Kaneshiro.

In the present paper, we report on sequential changes in sexual behavior among six populations of *Drosophila silvestris* (Perkins) from the island of Hawaii. It will be shown that data obtained from this study extends the interpretation of Carson and Bryant (1979) to an even finer level of differentiation in *silvestris*.

MATERIALS AND METHODS

During the initial stages of this study, strains of *silvestris* from four sites on the island of Hawaii were established. Offspring from single females captured from these sites were used to establish each stock. These four sites are numbers 1, 2, 4, and 5, as listed in Table 1. The stocks established from these four localities were used as standards for subsequent experiments with strains from localities 3 and 6, as given in Table 1.

The behavioral experiment used was the same as described in Kaneshiro (1976) and Ohta (1978). Since the females from the different strains were morphologically indistinguishable, a small spot of white enamel was placed on the mesonotum of one of the females after cooling them at 0°C for 4–5 min to immobilize them (Ohta 1978). Although it was determined from earlier experiments that the marking and cooling procedure had no effect on mating preference of the females, the females to be marked were alternated throughout the study to eliminate any possibility for bias in the data. After a maturation period of 1 month, two virgin females—for example, one from strain A and one from strain B—were placed in a vial with a sexually mature male from the same strain as one of the females—for example, strain A. The reciprocal combination (i.e., a male from strain B with two females from strains A and B) was also studied. Up to 100 such trios could be observed simultaneously. Each trio was observed for 3 hr (from 8 : 00–11 : 00 AM) each morning until one of the females mated with the male, and the number of homogamic and heterogamic matings was recorded. Length of copulation averaged 6–8 min. Since it had been shown (Kaneshiro 1976) that *silvestris* will not mate in total darkness, vials of trios that had not mated at the end of a morning observation period were covered with a black cloth until 8:00 the next morning. Unmated trios were observed for a period of 2 weeks,

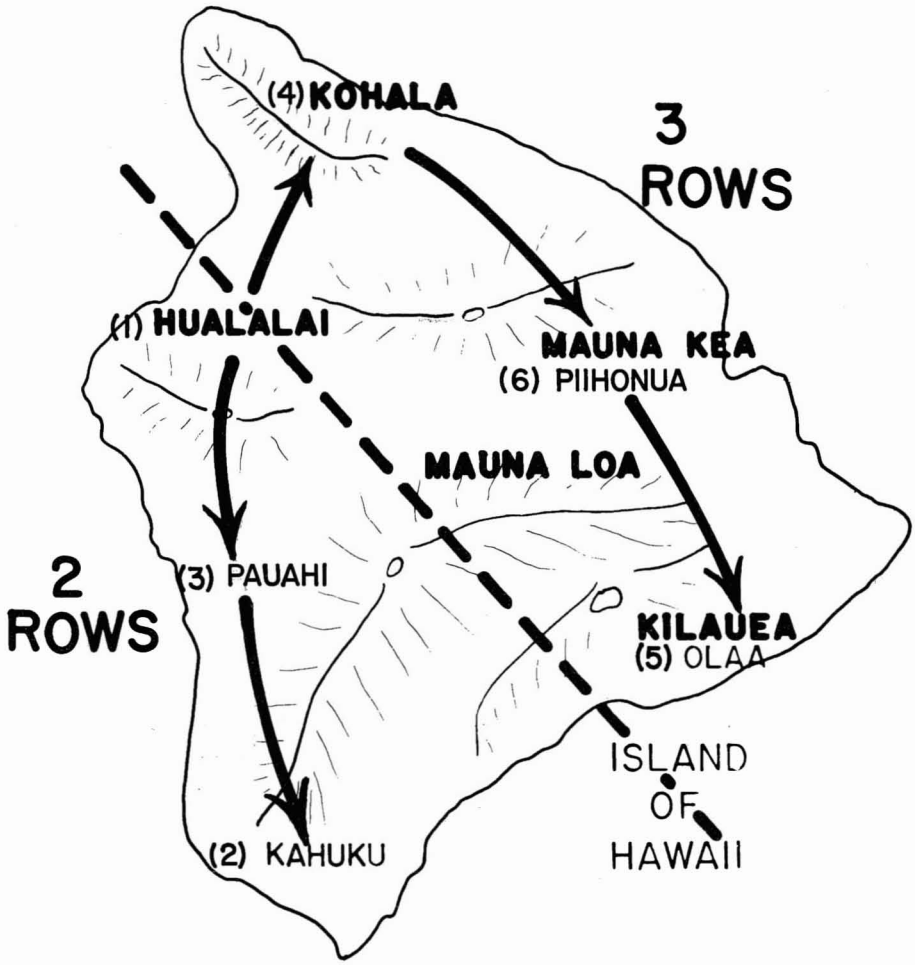

FIGURE 1. Map of the island of Hawaii showing the localities of the six *Drosophila silvestris* populations studied, and the sequence of colonization of these six populations.

after which those still with unmated females were discarded.

When the data for the initial four strains were collected and combined with the information of the morphotypes discovered by Carson and Bryant (1979), a pattern emerged that required the testing of two more populations of *silvestris* (from sites 3 and 6 in Table 1). These two sites are located approximately halfway between sites 1 and 2 and between sites 4 and 5 (see Figure 1). The populations from these two localities were tested only against adjacent populations. That is, male-choice experiments were conducted between strains from site 1 and site 3, sites 2 and 3, sites 4 and 6, and sites 5 and 6. The strains from sites 1, 2, 4, and 5 were used as standards against which the F_1 offspring of several isofemale lines from sites 3 and 6 were tested. Table 1 gives the number of isofemale lines used for these two sites.

The Charles–Stalker index (Stalker 1942) for sexual isolation was calculated for each reciprocal combination. The index (I) is calculated as the frequency of homogamic matings minus the frequency of heterogamic matings, divided by the sum of the frequencies (in our case, the sum of the frequencies is

TABLE 2

MATE PREFERENCE EXPERIMENTS AMONG SIX POPULATIONS OF *Drosophila silvestris*

♂	♀♀	TOTAL MATINGS	FREQUENCY HOMOGAMIC	FREQUENCY HETEROGAMIC	I*	C†
Hualalai (H)	H Pa	36	0.31	0.69	−0.38	−2.28
Pauahi (Pa)	Pa H	35	0.80	0.20	+0.60	+3.55
Haulalai (H)	H Ka	35	0.14	0.86	−0.72	−4.26
Kahuku (Ka)	Ka H	33	0.82	0.18	+0.64	+3.68
Pauahi (Pa)	Pa Ka	33	0.30	0.70	−0.40	−2.30
Kahuku (Ka)	Ka Pa	37	0.86	0.14	+0.72	+4.38
Hualalai (H)	H Ko	39	0.46	0.54	−0.08	−0.50
Kohala (Ko)	Ko H	35	0.71	0.29	+0.42	+2.48
Kohala (Ko)	Ko Pi	51	0.47	0.53	−0.06	−0.43
Piihonua (Pi)	Pi Ko	41	0.59	0.41	+0.18	+1.15
Kohala (Ko)	Ko O	40	0.23	0.77	−0.54	−3.42
Olaa (O)	O Ko	42	0.69	0.31	+0.38	+2.46
Piihonua (Pi)	Pi O	38	0.29	0.71	−0.42	−2.59
Olaa (O)	O Pi	37	0.68	0.32	+0.36	+2.19

*Charles–Stalker isolation index.

†$C = 2\sqrt{n}(p - 0.5)$; at the 5 percent confidence interval, the null hypothesis that mating is random is accepted when $-1.96 < C < +1.96$.

always 1 since we include only those replicates in which only one of the females is mated). A test of proportions (C) was calculated for each of the two reciprocals to test the null hypothesis that the two females in each vial mated at random. The value of C was calculated by using the formula $C = 2\sqrt{n}(p - 0.5)$ (Woolf 1968), where n is the total number of homogamic and heterogamic matings for each reciprocal and p is the frequency of homogamic matings. At the 5 percent level, the null hypothesis that the two populations are mating at random is accepted when $-1.96 < C < +1.96$.

RESULTS

In all except three combinations, the null hypothesis that matings between the two populations are random was rejected (Table 2). In the combinations involving Hualalai and Kohala females in vials with Hualalai males, and in the two reciprocals involving the Kohala and Piihonua populations, the isolation indices are low and the C values are not significant at the 5 percent level. Nevertheless, in these three combinations, as well as in all the others, the isolation is asymmetrical between the reciprocals. In all cases, the isolation index for one reciprocal is positive, indicating an excess of homogamic matings, while the index for the other reciprocal is negative, indicating an excess of heterogamic matings. Although the combination involving Hualalai males with Hualalai and Kohala females has a C value that indicates the females from the two populations accept the Hualalai male equally well, in the reciprocal combination involving the same females with Kohala males, the C value indicates the Hualalai females strongly discriminate against Kohala males. Even in the two reciprocals involving the Kohala and Piihonua populations where the statistical analyses indicate that the two populations show no discrimination against each other, there appears to be at least a tendency for asymmetry. Although males from Kohala mate nearly at random with either the Piihonua or their own females, males from Piihonua appear to be somewhat less successful in being accepted by Kohala females.

As mentioned earlier in this paper, Carson and Bryant (1979) discovered a new morphological character that may be used to differ-

entiate qualitatively between the southern and western populations of *silvestris*. Thus, except for the experiments involving the Kohala and Hualalai populations, all the other combinations involved populations that were either of the two-row morphotype or the three-row morphotype and not between the two types.

Of the two-row populations, Hualalai females strongly discriminated against males from both Pauahi and Kahuku. Pauahi females discriminated against Kahuku males but accepted Hualalai males very well. Kahuku females, on the other hand, accepted males of both the Hualalai and Pauahi populations very well.

On the other side of the island, where the populations have three rows of cilia on the front tibia, Kohala females strongly discriminate against Olaa males but only show a tendency for discrimination against Piihonua males. Piihonua females strongly discriminate against Olaa males but readily accept Kohala males. Olaa females accept males from both Piihonua and Kohala. In the experiments involving the two-row population from Hualalai and the three-row population from Kohala, females from Hualalai strongly discriminate against males from Kohala, but females from Kohala readily accept males from Hualalai.

DISCUSSION

The findings of Carson and Bryant (1979) and the idea that populations of *silvestris* on the north and east side of Hawaii are derived populations corroborate the interpretations of the changes in sexual behavior among these populations. By applying the hypothesis proposed by Kaneshiro (1976), it appears that the most ancestral of the two-rowed populations is confined to Hualalai, while the most ancestral of the three-rowed population is found on the Kohala Mountains. When these two crucial populations are experimentally compared, the indication is that the Hualalai population is the most ancestral; i.e., females from the Hualalai population discriminate against males from the Kohala population, while females of the Kohala population readily accept males of the Hualalai population as well as their own. It appears that despite the fact that the Kohala Mountains are geologically older than Hualalai, the most ancestral populations of *silvestris* occur on Hualalai. Subsequent to the colonization on Hualalai and the origin of *silvestris*, two separate lineages colonized the other areas where *silvestris* occurs (Figure 1). One lineage went south from Hualalai down to the southern slopes of Mauna Loa where the Kahuku Ranch population occurs. The other lineage went northward from Hualalai to the Kohala Mountains where the three-row population originated. From Kohala, the populations at Piihonua in the saddle between Mauna Kea and Mauna Loa and at Olaa on Kilauea were founded.

The above interpretation of the experimental behavioral data among the six populations of *silvestris* seems to corroborate the conclusion proposed by Carson and Bryant (1979) that the extra row of cilia on the front tibia of *silvestris* males is an incipient morphological character that arose as a result of changes in the courtship behavior pattern of *silvestris*. The behavioral data further indicate that such a shift first made its appearance in the Kohala population. Subsequently, colonizations of the Piihonua and Olaa populations from Kohala retained the new secondary sexual character.

The results of this study clearly suggest the role of behavior, especially that involved in sexual selection, in mechanisms leading to speciation. Behavioral differentiation in the form of partial (asymmetrical) sexual differentiation is evident among the six populations of *silvestris* studied despite the high degree of chromosomal and allozymic similarity between the two subdivisions of the species (Carson and Bryant 1979, Craddock and Johnson 1979, Sene and Carson 1977). The data indicate susceptibility to shifts in behavioral patterns due to the genetic reorganization that accompanies founder events and/or genetic drift situations. Furthermore, stepwise differentiation of sexual behavior occurs within each of the two (north and south) morphotypes of *silvestris* as diagnosed by the

tibial cilia of the males. Because of this, the behavioral data are the most sensitive indicators of newly acquired differentiation among populations.

Research is continuing in an attempt to determine precisely which components of the mate recognition system may be responsible for the behavioral differentiation observed among the six populations of *silvestris* and, perhaps, to determine how the new morphological structure was acquired. Such studies should provide further insights into the mechanisms of speciation.

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